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(71) Applicant: ROCKEFELLER UNIVERSITY
1230 York Avenue
New York, NY 10021(US)

(72) Inventor: Lizardi, Paul
500 East 63 Street
New York New York 10021(US)

(72) Inventor: Nogueira, Nadia
6 Greenholm
Princeton New Jersey 08540(US)

(74) Representative: Patentanwälte Schulze Horn und Hoffmeister
Goldstrasse 36
D-4400 Münster(DE)

(54) **Nucleotide hybridization assay for protozoan parasites.**

(57) The present invention provides a method for detection of protozoan parasites in blood or other specimen from their mammalian hosts. The method comprises nucleic acid hybridization of repetitive nuclear DNA fragments of the parasites. Hybridization probes have been prepared for this purpose by cloning the repetitive nuclear elements that are species-specific in appropriate vectors. The sensitivity of these probes has been increased by further sub-cloning to make them capable of cascade hybridization. The assay is highly specific and sensitive for detection of disease-causing protozoan parasites of the commonly occurring Trypanosomatidae of the genus *Leishmania* and genus *Trypanosoma*, as well as for malaria-causing protozoan and other parasitic microorganism of mammals.

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This invention concerns nucleic acid hybridization assays of biological samples for protozoan parasites utilizing hybridization probes constructed from species-specific repetitive nuclear DNA. The high senstitivity of these assays make them suitable for analysis of blood and other tissue specimen.

Background

Protozoans which invade the blood stream and tissues of higher animals and live parasitically therein may cause serious diseases in the host. In fact, many of the diseases that have plagued mankind for centuries are due to protozoan parasites. African sleeping sickness, for example, is generally caused by *Trypanosoma brucei gambiense* which is spread by the bite of the tsetse fly, a blood-sucking insect. Another species of this genus, *Trypanosoma brucei brucei* infects domestic animals; death follows infection for horses, cattle and pigs. Kala-azar is a serious disease caused by *Leishmania donovani* which is spread by sand-flies. Chagas disease is wide-spread in tropical areas where *T. cruzi* infections occur.

A sensitive, rapid assay is needed for diagnosis of these diseases. Current techniques involve the isolation and cultivation of individual parasites which is time-

consuming and often un-reliable (Chance, M. L., et al. Ann. Trop. Med. Parasitol. 68: 307 (1974); Martin, E., et al., Protozoology 23: 600 (1978); Miles, M. a., et al., Trans. R. Soc. Trop. Med. Hyg. 74, 243 (1979); Lainson, R., Trans. R. Soc. Trop. Med. Hyg., 75: 530 (1981)). Lesions due to infection by Leishmania species may be analyzed for parasites, by direct examination but this method does not allow identification of species causing more serious forms of the disease. Moreover other species, such as Tripanosomae do not express themselves in lesions and so require sensitive serological tests. The more reliable of those are difficult to use in large scale (immunofluorescence), or display cross-reactivity with other microorganisms (complement fixation, agglutination).

In addition, a major health problem has been created in areas of high infection when blood is collected for transfusion purposes. Since blood is a carrier of the parasites, blood from an infected individual may be unknowingly transferred to a healthy individual. Chagas disease, particularly has been found to be a major problem for blood banks in countries where the disease is endemic, with many documented cases of transmission of the infection by blood transfusions. Current assays for these protozoan parasites have neither specificity nor sensitivity to detect parasites in blood and are thus not useful for screening of blood to be used for transfusion.

The economic toll to agriculture due to these protozoan parasites of domestic animals is immense. Infection by Tripanosomae brucei, for example, is fatal to cattle unless treated early in the course of disease. There is no currently available sensitive diagnostic assay for infection, so many animals are lost. Moreover, the effectiveness of chemotherapy in both humans and animals

cannot be currently monitored so there is often needless reccurrence of the disease.

Wirth and Pratt (Proc. Nat'l. Acad. Sci. USA 79 6999 (1983) have described a hybridization assay for the detection of Leishmania parasites using probes to parasite kinetoplast DNA. This assay detects parasites in cutaneous lesions at a sensitivity level of 1,000 - 10,000 parasites per biopsy specimen. The specimens are collected by touch-blotting of nitrocellulose sheets over a small area of infected skin. However, this method is not sensitive enough to detect small numbers of parasites and relies on probes that have to be purified from the parasites themselves. This requires growth of these organisms in large quantities in the laboratory. Moreover, the method cannot be extended to parasites other than Trypanosomatidal, since these are the only ones to possess a kinetoplast.

A more sensitive assay has been sought for both Leishmania and other protozoan parasites which will be sensitive, specific, and thus be useful in early diagnosis of infection, will identify of species of parasites more likely to induce severe disease, and aid evaluation of chemotherapy and screening of blood bank samples.

The complementary nature of the double-stranded DNA which comprises the genome plays a fundamental role in the duplication of the cell and in the transcription and translation of genetic information. In the laboratory, complementary strands of DNA may be readily dissociated, and under appropriate conditions of cation concentration, and fragment size, may be reproducibly re-associated. This complementary nature of DNA is the basis of a sensitive assay for genetic material. The southern blot method, for instance, utilizes hybridization probes which are complementary DNA strands. A realated assay,

the Northern blot method, utilizes the ability of DNA to associate with complementary RNA probes. Detection devices employing RNA or DNA tagged with either a radioactive element or biotin have been developed (Gardner, L., Biotechniques March Volume 38 (1983); langer, P. R., et al., Proc. Natl., Acad. Sci. U. S. A., 78 6633 (1981).

The reproducibility and reliability of the blot methods in general is due to the accuracy with which complementary strands recognize one another. However, each genome contains a large number of nucleotide pairs. The calf genome, for example, contains 3.2×10^{19} nucleotide pairs (McCarthy, B. J., Progr. Nucleic Acid Res. Mol. Biol. 4, 129 (1965). It might seem that the probability of one chain finding and combining with its complement would be small. Surprisingly, however, it has been found that in genomes of some organisms the DNA occurs in repeated sequences that may account for a large proportion of the total genome (Britten, R. J. et al., Carnegie Inst. Wash. Year Book, 66, 73 (1967); ibid 65, 73 (1966)). Ten percent of the mouse genome, for example, consists of a million copies of a short nucleotide sequence (Waring, M. et. al., Science, 154, 791 (1966)). It has been observed that many other species also contain repetitive DNA elements (Britten et al., Science 161, 529 (1968)).

Since the blot hybridization methods detect DNA sequences, it follows that sesitivity and specificity of assays are enhanced if a probe which recognizes a repetitive sequence can be obtained. Moreover, if this repetitive sequence is species specific, the probe recognizing it is also specific. Protozoan parasites containing such species-specific repretitive nuclear DNA elements have been sought.

SUMMARY

The present invention provides a method for detection of protozoan parasites in blood or other specimen from their mammalian hosts. The method comprises nucleic acid hybridization of repetitive nuclear DNA fragments of the parasites. Hybridization probes have been prepared for this purpose by cloning the repetitive nuclear elements that are species-specific in appropriate vectors. The sensitivity of these probes has been increased by further sub-cloning to make them capable of cascade hybridization. The assay is highly specific and sensitive for detection of disease-causing protozoan parasites of the commonly occurring Trypanosomatiade of the genus <u>Leishmania</u> and genus <u>Trypanosoma</u>, as well as for malaria-causing protozoan and other parasitic microorganism of mammals.

DETAILS

We have discovered that protozoan parasites conatin species-specific repetitive nuclear DNA elements.

The present invention provides a method for detection of protozoan parasites comprising nucleic acid hybridization with hybridization probles constructed from these species-specific repetitive nuclear DNA fragments.

The invention may be understood more fully in view of the accompanying Figures of which

Figure 1 illustrates the specificity of the <u>T. cruzi</u> repetitive element; and

Figure 2 illustrates the detection of parasites in the blood of humans infected with <u>T. cruzi</u>.

Figure 3 illustrates the analysis of cloned *P. falciparum* DNA inserts by agarose gel electrophoresis.

Figure 4 illustrates the nucleotide sequence of the repetitive DNA from clones pPFR-1 and pPFR-5.

Figure 5 illustrates the detection of *P. falciparum* DNA from blood samples using dot blot hybridization.

Availability of DNA Hybridization Probes

Clones comprising repetitive nuclear DNA fragments specific for protozoan parasites disclosed in the present invention are deposited with The Rockefeller University, 1230 York Avenue, New York, New York 10021. Preferred clones of the present invention comprising repetitive nuclear DNA fragements specific for *T. cruzi* and for *P. falciparum* are also deposited at the American Type Culture Collection, Bethesda, Maryland and bear the following deposit numbers:

Clone #	ATCC #
pTC-NRE	40078
pTC multi-NRE	40077
pPFR-1	39619
pPFR-5	39618

Deposit is for the purpose of enabling disclosure only and is not intended to limit the concept of the present invention to the particular materials deposited.

Isolation Identification and Synthesis of Species-Specific Repetitive Nuclear DNA Fragments

Repetitive nuclear DNA fragments have been isolated from protozoan parasites by the following method:

a. Parasite DNA was sheared by sonication to an average size of 1,500 - 2,000 base pairs, and then denatured with alkali.

b. The DNA solution was neutralized, and the single strands were allowed to renature to a Cot of 2.0 (Balton et. al., Carnegie Inst. Wash. Year Book, 65, 78 (1965)).

c. The rapidly-renatured (Cot 2.0) DNA fraction was treated with S1 nuclease to destroy residual single-stranded DNA.

d. The repetitive DNA was made blunt-ended by treatment with Klenow DNA polymerase I, and was then joined to Bam HI linkers (Deininger, P. et al., J. Mol. Biol. 151, 17 (1981)). This DNA was then ligated to a suitable vector (PUC 13 or pBR 322) and used to construct a library of repetitive DNA elements. The library was stored in multiple replicas (nitrocellulose filters containing 1000 colonies/filter).

e. A DNA probe from the same parasite was prepared by nick-translation (2) of total parasite DNA. Other probes are prepared by nick-translation of total DNA from other parasites or from vertebrate hosts of interest. Whole DNA should be screened for cross-reactivity with the clones from the primary parasite.

f. Identical library replicas containing repetitive DNA clones (500 - 1,000 colonies/filter) were hybridized

as follows:

- With labeled total DNA from the same parasite. Hybridization for a short period of time (1 hr.) is used to identify clones with the highest repetitivity.

- With labeled DNA from other parasites, or from vertebrate hosts of interest. Hybridization is allowed to proceed to completion ("4 hour) to achieve maximum sensitivity.

g. Clones were selected which give maximum hybridization signal with the primary parasite, and yet show no evidence of cross-reactivity.

h. The clone was modified, if necessary, by addition of extra repetitive DNA of the same kind or a combination of repetitive DNAs from other genes to insure that the resulting probe will be capable of cascade hybridization.

i. Combination probes may also be constructed to comprise a tandem linear assay of nuclear and/or kinetoplast repetitive elements.

j. The probe was tested in hybridization assays similar to those described for T. cruzi and P. falciparum.

The method outlined in above have been applied to the parasites Leischmania brasiliensis, L. Tropica, L. mexicana, and L. donovani, T. cruzi, T. brucei brucei, T. brucei gambiense and Plasmodium falciparum and may be applied to other protozoan parasites as well.

Repetitive DNA fragments from other protozoan parasites of the same genus and from related geni may be isolated

by the same and similar methods. Examples include, but are not limited to Trypanosomatidae parasites of the genus *Leishmania* and *Trypanosoma*, as well as organisms of the genus *Plasmodi* and *Babesia*. *Leischmania* parasites, for example, comprise *L. donovani, L. brasiliensis, L. tropica*, and *L. mexiana*. *Trypanosomae* may be *Salivarian* or*Stercorarian*. Salivarian trypanosomes comprises *T. bruckei, T. congolense*, and *T. vivax*. Stercorarian tripanosones comprise *T. cruzi*. Also included are filarial parasites comprising *Wuchereria Bancrofti* and *Brugia malayi*, and the human malaria-causing parasites *P. malaria, P. vivax.* DNA from the commonly occurring blood parasites of mammals and amoebae from feces may also be isolated by the given method or others known in the art and used as kybridization probes in the assays of the present invention.

The repetitive nuclear DNA element isolated from *T. cruzi* has been analyzed for nucleotide sequence and found to be as follows:

CTCTTGCCCACACGGGTGCTGCACTCGGCTGATCGTTTTCGAGCGGCTGC
TGCATCACACGTTGTCGTCCAAATTTTTGTTTCCGATTGTGAATGGTGGC
AATCGGAAACACTCTCTCTGTCAATATCTGTTTGCGTGTTCACACACTGGACACCA
AACAACCCTGAACTATCCGCTGCTTGGAGGAATTTCGCGAG

This DNA element exhibits micro-variations. However, although this sequence varies 5 - 20 % at individual positions, it still is a species-specific hybridization probe for *T. cruzi*. It is to be understood that the hybridization probes ob the present invention comprise these variations.

Six independent clones of *P. falciparum* DNA have been isolated using the plasmid PUC13 as vector. The clones, which have been named pPFR-1 through pPFR-6, contain inserts with sizes ranging from 280 to 1200 base pairs

(BP), approximately. The cloned inserts are displayed in Figure 1 as stained DNA bands in an agarose gel. Table I shows the approximate sizes of each of the cloned inserts. The table also shows that clones 3, 4, and 5 belong to the same sequence family. Clones 1, 2 and 6 are all different from each other and from the C. family. The abundancy of C-family clones suggests that they represent the major repetitive element of P. falciparum.

The DNA of clones pPFR-1 and pPfr-5 has been sequenced. Figure 2 shows that the basic repeating unit of pPFR-1 is a 51 bp sequence, while for pPFR-5 the repeating unit is 21 bp. The repeating units show small imperfections, or sequence microheterogeneities.

Table 1

P. FALCIPARUM REPETITIVE DNA CLONES

	Insert size	Sequence Family*
pPFR-1	1200	A
pPFR-2	800	B
pPFR-3	280	C
pPFR-4	320	C
pPFR-5	340	C
pPFR-6	950	D

*Sequence family relationships were determined by hybridization of labeled DNA from each clone with Southern blots containing DNA from all six inserts.

The DNA sequence may be synthesized by methods well known in the art either chemically or biologically. DNA complementary to this sequence and RNA having the same or complementary sequence may be similarly synthesized.

DNA sequences may also be constructed which consists of repeats of the repetitive element, or repeats of a subdomain of the element. A third possibility is the construction of mixed element clones, containing tandem repeats of various elements (a, b, c ...) in the form a-a-a ... - b-b-b ... c-c-c ... . Likewise, multi-a multi-b, and multi-c clones may be constructed independently, and mixed at the time of the tagging reaction.

When tagged with radioactive element preferrably ^{32}P, or biotinylated, the natural or synthesized repetitive DNA and RNA are useful in the hybridization assay of the species from which they were first isolated. These repetitive DNA elements do not appear to be from structural genes nor coded for any known structural products such as proteins.

Preparation of hybridization probes

Hybridization probes for protozoan parasites may be prepared by cloning the nuclear repetitive elements that are species-speciefic in a suitable vector and labeling with a suitable label.

Vectors into which the DNA element may be inserted and replicated are suitable. Such vectors comprise, for example, *E. coli* plasmids, filamentous phages, lamboid bacteriophage, cosmids and ceast shuttle vectors. Other vectors known in the art may be employed. Especially preferred is the plasmid pUC 13.

For purposes of the DNA hybridization assay the clones are labeled with either a radioactive element or a chromagen such as biotin. The clones maybe labelled, for example, with ^{32}P by nick-translation with DNA polynerase in the presence of ^{32}P-dcTP (Botchan, M., et.

al., *Supra*).

Alternatively, in assays utilizing RNA hybridization, tagged RNA complementary to the repetitive DNA is prepared.

Appropriate promoter sequences may be fused to the DNA of interest and replicated in an appropriate host such as *E. coli* using standard cloning techniques. This DNA may then be isolated from the *E. Coli* and incubated *in vitro* with RNA polymerase to generate radioactive or biotinylated RNA. The method of Green, M. et al., (Cell **32**, 681 (1983)), for example, may be used to generate biotinylated or radioactive RNA complementary to the repetitive nuclear DNA.

The hybridization probes containing species-specific nuclear repetitive DNA may be further modified by addition of extra repetitive DNA to ensure that the resulting probe is capable of cascade hybridization in DNA/DNA hybridization assay. Because of the formation of multiple-concatenamer DNA networks due to the cascade hybridization signal, the sensitivity of the assay is significantly increased. The hybridization probes with cascade signal may detect DNA equivalent to as little as one parasite because the hybridization signal is amplied. This increased sensitivity makes the assay suitable for early diagnosis of infection or for blood bank screening and useful in the monitoring of chemotherapy directed at eliminating known infection.

Nucleic Acid Hybridization Assay

The assay for protozoan parasites may be performed on any biological sample suspected of containing the parasite. Blood, for example, or biopsy tissue or matter

from lesions obtained by blot or wash are suitable samples for assay. Because of its sensitivity, the method of the present invention may be applied to blood where as little as one parasite per specimen may be detected.

In the method of the present invention, the parasite DNA isolated from a tissue biopsy specimen or body fluid by phenol extraction (Lizardi, P. et al., Methods in Enzymology, Vol. 96 "Biomembranes" Eds. S. Fleisher and B. Fleisher; Academic Press, N. A.) or a small tissue biopsy, body fluid or parasite culture sample which can be efficiently solubilized in alkali (without prior phenol extraction) is spotted on nitrocellulose or similar solid support such as gene-screen using dot-blot methodology (Kafatos et al., Nucleic Acids Research, 7, 1541 (1979)). After DNA binding, the nitrocellulose or other solid support is contacted with radioactive probe under conditions suitable for reaction between probe and bound DNA. The nitrocellulose, for example, maybe incubated in the presence of hybridization solution (Kafatos, et al., Supra) at an appropriate temperature, about 46^{o} for about 4 to 20 hours. The nitrocellulose is removed from solution, washed, and, when a radioactive probe has been applied, is exposed radioautographically using X-ray film and intensifying screens. A signal is observed as dark spots on the film after 4 - 24 hour exposure. When the probe is biotinylylated the nitrocellulose is exposed to an avidin-enzyme system and observed for colored spots indicative of parasite DNA.

The specificity of the assay may be tested by contacting other protozoan parasites with the probe. Control samples of known parasite DNA may be run concurrently for comparison.

The invention may be illustrated by the following examples, but is not intended to be limited thereby.

Example 1

This example illustrates the isolation and identification of the repetitive nuclear DNA element from T. cruzi. T. cruzi epimastigote DNA was cut with a variety of restriction enzymes. Digestion with SST I generated a coherent band of abosut 200 base pairs which contained seven to ten percent of the total parasite DNA. The element contains an 195 base pair domain bounded by SST I sites, but the actual length of the repetitive sequence may be somewhat longer. Southern blot analysis using the radioactive repetitive probe showed that at least some of the repetitive elements occur in tandem domains. Since this repetitive element is repeated about 100,000 times in the T. cruzi genome, it provides the basis for a hybridization assay capable of detecting a fraction of a picogram of parasite DNA.

The sequence was used to derive the expected thermal stability (Tm) of the DNA. The Tm of 90.4^{o} C identified this DNA as being of nuclear origin, since kinetoplast DNA is known to have a much lower Tm.

Example 2

This example illustrates the preparation and specificity of hybridization probes capable of genereating a cascade hybridization signal in a DNA/DNA hybridization assay.

The repetitive nuclear DNA element from T. cruzi (Example 1) was isolated after separation from the digestion medium by a 1.5% agarose gel and cloned in the plasmid pUC 13 (Vieira, J., et al., Gene 19, (1983)). The clone was named pTCNRE (plasmid T. cruzi - nuclear repetitive element). Other clones were constructed which

contained not one, but several copies of the element. These new clones were termed pTC-multi NRE and are capable of generating a hybridization signal which will be amplified about 40 fold by cascade hybridization.

Hybridization probes were constructed by labeling pTCNRE by nick-translation with DNA polymerase in the presence of ^{32}P-dcTP (Botchan, M., et al. Supra) yielding a specific activity of 10^{8} CPM per microgram.

The *T. cruzi* probe hybridized with all of the eight *T. cruzi* strains tested. Hybridization was negative with mouse or human DNA.

Figure 1 illustrates the specificity of the *T. cruzi* hybridization probe. The probe detected *T. cruzi* in epimastigotes and the blood stream forms, but was negative for *L. donovani*, *L. tropica*, *L. braziliensis*, *L. mexicana*, *T. evansi*, *T. equiperdum*, *T. brucei brucei*, *T. lewisi* and *T. congolense*.

Example 3

This example illustrates the assay of blood for *T. cruzi.*

T. cruzi DNA (isolated from epimastigotes by phenol extraction) was spotted on nitrocellulose using dot-blot methodology (Kafatos, et al., Supra). This technique, which is well described in the cited reference simply involves denaturation of DNA in alkali, neutralization, and spotting on nitrocellulose in the presence of high salt (NaCl). Amounts of *T. cruzi* DNA ranging from 0.01 ng (equivalent to 30 organisms) were spotted to provide a series of standards. Two kinds of experimental samples can also be spotted on nitrocellulose: (a) Any kind of DNA obtained from a tissue biopsy or body fluid by

phenol extraction. (b) Any small tissue biopsy, body fluid, or parasite culture sample (1 ul to 5 ul) which can be efficiently solubilized in alkali (without prior phenol extraction). Samples to be bound are conveniently organized using a 8 x 12 well Hybry-dot apparatus (Schleicher and Schuell, Inc., Keene, New Hampshire) which aligns spots of about 5mm diameter on the nitrocellulose. After DNA binding, the nitrocellulose was baked for 2 hours and then placed in a plastic bag containing hybridization solution (3). Radioactive probe pTC NRE (from Example 3) was added, the bag was sealed, and incubated at the appropriate temperature 46^{o} C for 4 - 20 hours. The nitrocellulose was then taken out of the base and washed several times in low salt-sodium dodecyl sulfate solution. Finally, it was exposed radioautographically using X-ray film and intensifying screens. A signal was observed as dark spots in the film after 4-24 hour exposure.

The assay with pTC-NRE plasmid detected DNA equivalent to about 30 parasites. The pTC multi-NRE plasmids detect DNA equivalent to 1 parasite because the hybridization signal is amplified about 40-fold by cascade hybridization (formation of multi-concatenamer DNA networks).

The pTCNRE assay is species-specific. Tests with different *T. cruzi* strains have been positive with all strains tested. The sensitivity of the assay was found to be sufficient to detect one *T. cruzi* cell in a background of o.3cc blood. The results of assay of blood of five individuals throught to be infected with *T. cruzi* are illustrated in Figure 2. All five individuals showed positive hybridization whereas normal human blood gave negative results.

Examples 4 - 7

The method of Example 3 may be used to assay blood for *Leishmania brasiliensis*, *L. tropica*, *L. mexicana* or *L. donovani*. Blood, tissue biopsy or body fluids may be assayed by the method of Example 3 for any protozoan parasites.

Example 8

This Example illustrates a method of generating biotinylylated or radioactive RNA complementary to the species-specific repetitive nuclear DNA.

A piece of DNA containing Salmonella phage Sp6 promoter sequences is fused to the DNA clone of interest. This new DNA construction can be replicated in *E.coli* using standard cloning techniques. This DNA is then isolated from *E.coli*, and incubated *in vitro* with RNA polymerase (obtained from Salmonella phage SP6) and used generate radioactive or biotinylylated RNA.

Example 9

This example illustrates the method by which the species specificity of repetitive nuclear DNA elements from a particular protozoan parasite was established.

1. Total DNA was isolated from a protozoan parasite of interest and labeled by nick-translation. DNA from other parasites or from a vertebrate host was likewise isolated and labeled.

2. Repetitive DNA elements from the protozoan parasite was cloned in suitable vectors and affixed to

nitrocellulose to form library replicas upon which DNA were identically located. A first replica was hybridized with radio-active labeled DNA from the same parasite. A second replica was hybridized with labeled DNA from other paqrasites or from the vertebrate host.

3. The hybridized replicas were exposed to film for a time sufficient to produce development of radio-active areas corresponding to hybridization with labeled DNA.

4. The exposed films were superimposed and aligned in accordance with the location of clones in the replicas.

5. Many areas showed development in both replicas which indicated cross-reactivity between the species. However, some areas showed development in only the one replica containing the parasite of interest. This indicated species-specific DNA.

6. Those species-specific clones which gave maximum hybridization signal with the parasite of interest but not with other parasites or the host were selected and used as hybridization probes for this parasite.

Example 10

This example illustrates the use of DNA hybridization probes to detect P.falciparum in blood.

Clones pPFR-1 and pPFR-5 have been used in a hybridization assay to detect the presence of P.falciparum in blood. A mixture of the two DNA`s was labeled by nick-

translation and hybridized with a nitrocellulose sheet containing phenol-extracted DNA from infected as well as uninfected human blood. Known amounts of P.falciparum DNA were used as controls for quantiatiion. Figure 5 illustrates the results of these experiments.

The DNA standards show that the sensitivity of this assay corresponds to about 1 ng of P.falciparum DNA. With appropriate optimization the assay can detect 0.1 ng of DNA, which corresponds to the DNA content of $3.3x10^3$ parasites. This sensitivity is adequate to detect parasites in the blood of an infected patient, provided that the assay is carried out after the parasites have come out of the liver and entered erythrocytes. Typical blood parasitemia levels in human range from $4x10^3$ parasites/ml at very early stages up to $4x10^8$ parasites/ml in the acute stages. A level of 5,000 parasites per ml. would be easily detected by the hybridization assay, but would be virtually impossible to detect by conventional microscopic examination of blood (this level roughly corresponds to one infected erythrocyte for every 10^6 erythrocytes).

Compared to a light microscopic parasitemia count, this assay provides about 500-fold more sensitivity, and does not require an expert observer. The method is particuarly suitable for the testing of anti- P.falciparum chemotherapies. It is also ideally suited for the measurement of parasitemia in post-vaccination challenge experiments.

Example 11, 12

T.brucei gambiense

We have hybridized nitrocellulose filters containing copies of clone libraries from each of these two parasites. Using the methods described in Example 9, we have

isolated species-specific repetitive element clones have been frozen for further testing.

The clones in our possession include one clone containing DNA identical to that described by Sloof et al. (Sloof, et al., Nucleic Acids Res. 11, 3889 (1983)) hereinafter termed Sloof DNA incorporated by reference.

Examples 13, 14

We have existing frozen clone libraries containing repetitive elements (Kinetoplastic as well as not kinetoplastic) of *Leishmania tropica* and *Leishmania brasiliensis*.

Example 15

The methods of the present invention may be extended to other important parasites. The repetitive nuclear DNA elements may be isolated, identified as species-specific and used as hybridization probes as disclosed hereinabove. Examples include:

T. equiperdum
T. vivax *T. rhodesiense* *T. congolense* *Babesia boris* *Wuchereria bancroft* *Brugia malayi* *Entamoeba hystolitica* *Plasmodium malariae* *Theileric parva*

Figure 1: Specificity of hybridization of *T. cruzi* repetitive element

A_3, B_3 *T. cruzi* Y strain bloodstream forms
= 10^5 organisms*

A_6, B_6 *T. cruzi* Peru strain bloodstream forms
= 10^5 organisms*

C_5, D_5 *T. cruzi* Tulahuen strain epimastigotes
= 3×10^5 organisms*

C_6, D_6 *T. cruzi* Brasil strain epimastigotes
= 3×10^5 organisms*

E_3, F_3 *L. donovani* 50 ng DNA
E_4, F_4 *L. tropica* 50 ng DNA
E_5, F_5 *L. braziliensis* 50 ng DNA
E_5, F_6 *L. mexicana* 50 ng DNA
G_1 to G_5 *T. cruzi* DNA 50 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng
H_2 *T. evansi* = 1.5×10^5 blood stream forms
H_3 *T. equiperdum* "
H_4 *T. brucci brucei* "
H_5 *T. lewisis* "
H_6 *T. congolense* "

*Whole blood blottings

Figure 2. Detection of parasites in the blood of humans infected with *T. cruzi*.

A - 1 to 5 *T. cruzi* DNA 50 ng, 10, 1 and 0.1 ng

B - 2, 3 300 λ of total human blood from a normal subject

C_1 to C_5 300 λ of blood from patients with chronic Chagas' disease

1 - A. K. (from Parana) Brazil

2 - B. B. (city unknown) Brazil

3 - S. P. C. (Goias) Brazil

4 - M. J. G. (Minas Gerais) Brazil

5 - A. G. S. (Bahia) Brazil

Figure 3: Analysis of cloned P. falciparum DNA inserts by agarose gel electrophoresis

Plasmid DNA's were cut with the restriction endonucleases Eco RI and Hind III to release the cloned inserts. The DNA was then fractionated in a 3 % agarose pPFR-2, pPFR-3, pPFR-4, pPFR-5, pPFR-6, respectively. Lane 7 ØX DNA cut with Hae III (marker DNA fragments).

Figure 4: Nucleotide sequence of the repetitive DNA from clones pPFR-1 and pPFR-5.

The cloned insert in pPFR-1 is about 1200 bp long. We sequenced about 200 bp at each end and found a nearly perfect 51 bp repeat (see arrowheads). The insert is about 340 bp long and where we show a 129 bp domain corresponding to one side of the insert.

Figure 5: Detection of *P. falciparum* DNA from blood samples using dot blot hybridization.

DNA from the sources specified below was bound to nitrocellulose and hybridized with radioactive cloned DNA (a mixture of pPFR-1 and pPFR-5 DNA labeled by nick-translation). After washing unbound labeled DNA, the nitrocellulose was contacted with X-ray film and exposed for 16 hours using two intensifying screens.

A1 through A4, *P. falciparum* DBA, 10 ng, 1 ng, 0.1 ng, 0.01 ng, B1, DNA from normal human blood. B2 DNA from normal human blood which had been mixed with 1 ng. *P. falciparum* DNA. C1 through C4, and D1 and through D4, DNA from 10 µl of *P. falciparum* blood cultures. Dot intensities show good correlation with parasitemia counts.

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What is Claimed:

1. Method for detecting protozoan parasites in a biological sample from a vertebrate comprising contacting said sample with a labeled hybridization probe recognizing species-specific, repetitive nuclear DNA in said parasite and detecting said labeled probe bound to said parasite.

2. Method of Claim 1 wherein said hybridization probe comprises DNA or RNA having a nucleotide sequence corresponding to or complementary to all or a portion of said species-specific repetitive nuclear DNA.

3. Method of Claim 2 wherein said hybridization probe is DNA isolated from said parasite.

4. Method of Claim 2 wherein said hybridization probe is synthesized chemically.

5. Method of Claim 2 wherein said hybridization probe is synthesized from the component nucleotides in the presence of appropriate enzymes under conditions suitable reaction between said nucleotides.

6. Method of Claim 1 wherein said hybridization probe comprises DNA or RNA having a nucleotide sequence corresponding to or complementary to all or a portion of said species-specific repetitive nuclear DNA in an appropriate vector.

7. Method of Claim 6 wherein said vector is selected from the group consisting of *E. coli* plasmids, filamentous phages, lamboid bacteriphages, salmonella phages and yeast.

8. Method of Claim 7 wherein said vector contains salmonella SP6 phage promoter.

9. Method of Claim 7 wherein said *E. coli* plasmid is PUC 13.

10. Method of Claim 1 wherein said hybridization probe is capable of cascade hybridization.

11. Method of Claim 1 for detection of *T. cruzi* wherein said hybridization probe is capable of recognizing the approximately 200 base pair nuclear DNA fragment in *T. cruzi* nuclear genome.

12. Method of Claim 11 wherein said hybridization probe comprises said DNA or RNA in a suitable vector.

13. Method of Claim 12 wherein said vector is an *E. coli* plasmid.

14. Method of Claim 13 wherein said vector is PUC 13.

15. Method of Claim 1 for detection of *Plasmodium falciparum* wherein said pybridization probe is capable of recognizing the approximately 200 base pair nuclear DNA fragment in *plasnodium falciparum* nuclear genome.

16. Method of Claim 15 wherein said hybridization probe comprises said DNA or RNA in a suitable vector.

17. Method of Claim 15 wherein said vector is an *E. coli* plasmid.

18. Method of Claim 17 wherein said vector is PUC 13.

19. Method of Claim 1 for detection of _T. brucei gambiense_ wherein said hybridization probe is capable of recognizing Sloof DNA.

20. Hybridization probe recognizing species-specific repetitive nuclear DNA of a protozoan parasite.

21. Hybridization probe of Claim 20 labelled with a radioactive element or a chromophoric group.

22. Hybridization probe of Claim 21 wherein said chromophoric group is biotin.

23. Hybridization probe of Claim 20 comprising DNA having a nucleotide sequence corresponding to all or a portion of the species-specific repetitive nuclear DNA in said parasite.

24. Hybridization probe of Claim 23 wherein said DNA is isolated from said parasite.

25. Hybridization probe of Claim 23 wherein said DNA is chemically synthesized.

26. Hybridization probe of Claim 23 wherein said DNA is synthesized from the appropriate nucleotides in the presence of appropriate enzymes under conditions suitable for reaction between said nucleotides.

27. Hybridization probe of Claim 20 comprising DNA or RNA having the complementary sequence of all or a portion of the species-specific repetitive nuclear DNA in said parasites.

28. Hybridization probe of Claim 20 comprising DNA or RNA having a nucleotide sequence corresponding to or complementary to all or a portion of said species-

specific repetitive nuclear DNA in an appropriate vector.

29. Hybridization probe of Claim 28 wherein said vector is selected from the group consisting of E. coli plasmids, filamentous phages, lamboid bacteriophages, salmonella phages, and yeast.

30. Hybridization probe of Claim 29 wherein said vector contains salmonella SP6 phage promoter.

31. Hybridization probe of Claim 29 wherein said plasmid is PUC 13.

32. Hybridization probe of Claim 31 wherein said DNA, complementary DNA or RNA is in tandem array in said vector.

33. Hybridization probe of Claim 28 comprising repeated segements of said DNA, complementary DNA or RNA in said vector.

34. Hybridization probe of Claim 20 for Plasmodium falciparum comprising DNA having all or a portion of the sequence of the approximate 340 or 1200 bp sequenced nuclear DNA fragments of Plasmodium falciparum DNA or RNA complementary to all or a portion of said sequenced nuclear fragments.

35. Hybridization probe of Claim 34 in an appropriate vector.

36. Hybridization probe of Claim 35 wherein said vector is an E. coli plasmid.

37. Hybridization probe of Claim 45 comprising pTCNRE and pTC-multi NRE.

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38. Hybridization probe of Claim 34 wherein said nuclear DNA fragment has the sequence:
CTCTTGCCCACACGGGTGCTGCACTCGGCTGATCGTTTTCGAGC
GGCIGCTGCATCACACGTTGTCGTCCAAATTTTTGTTTCCGATTGTGAA
TGGTGGCAATCGGAAACACTCTCTGTCAATATCTGTTTGCGTGIICACACA
CTGGACACCAAACAACCCTGAACTATCCGCTGCTTGGAGGAATTTCGCGAG

39. Hybridization probe of Claim 38 wherein said sequence exhibits mini-variation of about 5 to 20 % at each base pair.

40. Hybridization probe of Claim 20 for <u>Plasmodium falciparum</u> comprising DNA having all or a portion of the sequence of the approximate 340 or 1200 bp sequenced nuclear DNA fragments of <u>Plasmodium falciparum</u> DNA or RNA complementary to all or a portion of said sequenced nuclear fragments.

41. Hybridization probe of Claim 40 in an appropriate vector.

42. Hybridization probe of Claim 41 wherein said vector is an <u>E. coli</u> plasmid.

43. Hybridization probe of Claim 41 comprising pPFR-1 and pPFR-6.

44. Hybridization probe of Claim 40 wherein said nuclear DNA fragment has the sequence:
TGTCCTCCAGACTTTTCTACCACTCGTAGAGTTTTCTGGGTACTGTGAACT
GACCTCCAGACTGATCTCTACAATCCGTAGAGTTTCTGGGTACTGTGAACT
GTCCTCCAGACTTTTCTACCACTCGTAGAGTTTCTGGGTACTGTGAACTGA
CCTCCAGACTGATCTCTACAATCCGTAGAGTTACTGGGTACTGTGAACTGA
CCTCC

45. Hybridization probe of Claim 40 wherein said nuclear DNA fragement has the sequence:

TTTTAGCTTTAGGGTTCAGGGTTTAGGGTTTAGGGTTCAGGGTTTAGGTT
TAGGGTTCAGGGTTCAGGGTTCAGGGTTTAGGTTTAGGGTTCAGGGTTTA
GGTTTAGGGTTCAGGGTTTAGGGTTTTCC

46. DNA having the nucleotide sequence:
CTCTTGCCCACACGGGTGCTGCACTCGGCTGATCGTTTTCGAGCGGCT
GCTGCATCACACGTTGTCGTCCAAATTTTTGTTTCCGATTGTGAATGGTG
GCAATCGGAAACACTCTCTGTCAATATCTGTTTGCGTGTTCACACACTGGA
CACCAAACAACCCTGAACTATCCGCTGCTTGGAGGAATTTCGCGAG
or fragments thereof.

47. DNA of Claim 46 isolated from T. cruzi.

48. DNA of Claim 46 synthesized in vitro chemically or ciochemically.

49. DNA or RNA complementary to DNA of Claim 46.

50. DNA of Claim 46 in an appropriate vector.

51. DNA having the nucleotide sequence:
TGTCCTCCAGACTTTTCTACCACTCGTAGAGTTTTCTGGGTACTGTGAACT
GACCTCCAGACTGATCTCTACAATCCGTAGAGTTTCTGGGTACTGTGAACT
GTCCTCCAGACTTTTCTACCACTCGTAGAGTTTCTGGGTACTGTGAACTGA
CCTCCAGACTGATCTCTACAATCCGTAGAGTTACTGGGTACTGTGAACTGA
CCTCC
or fragments thereof.

52. DNA of Claim 51 isolated from Plasmodium falciparum.

53. DNA of Claim 51 synthesized in vitro chemically or biochemically.

54. DNA or RNA complementary to DNA of Claim 51.

55. DNA of Claim 51 in an appropriate vector.

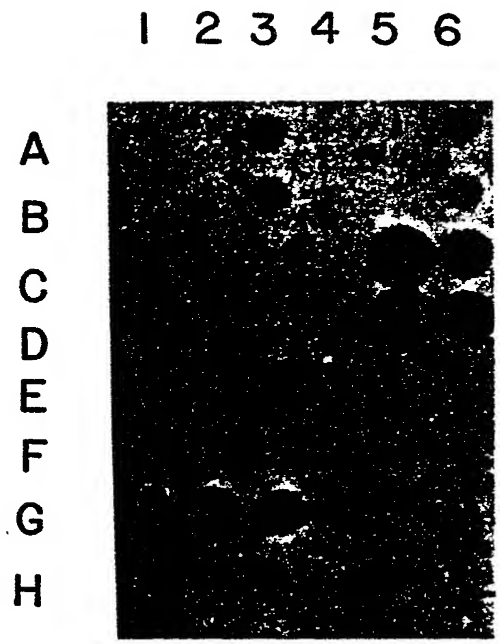
56. DNA having the nucleotide sequence:
TTTTAGGTTTAGGGTTCAGGGTTTAGGGTTTAGGGTTCAGGGTTTAGGTTTAGGGTTCAGGGTTCAGGGTTCAGGGTTTAGGTTTAGGGTTCAGGGTTTAGGTTTAGGGTTCAGGGTTTAGGGTTTTCC
or fragments thereof.

57. DNA of Claim 56 isolated from <u>Plasmodium falciparum</u>.

58. DNA of Claim 46 synthesized <u>in vitro</u> chemically or biochemically.

59. DNA or RNA complementary to DNA of Claim 56.

60. DNA of Claim 56 in an appropriate vector.



Specificity of hybridization of _T. cruzi_ repetitive element.

FIG. 1

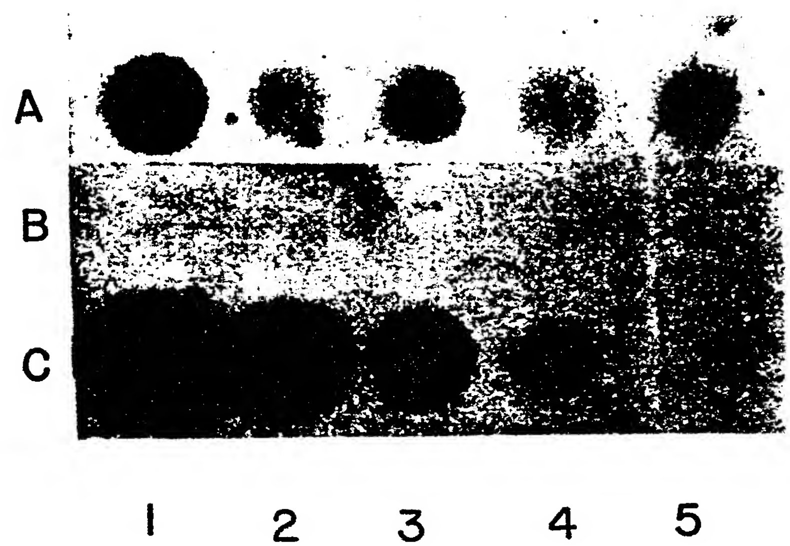


FIG. 2

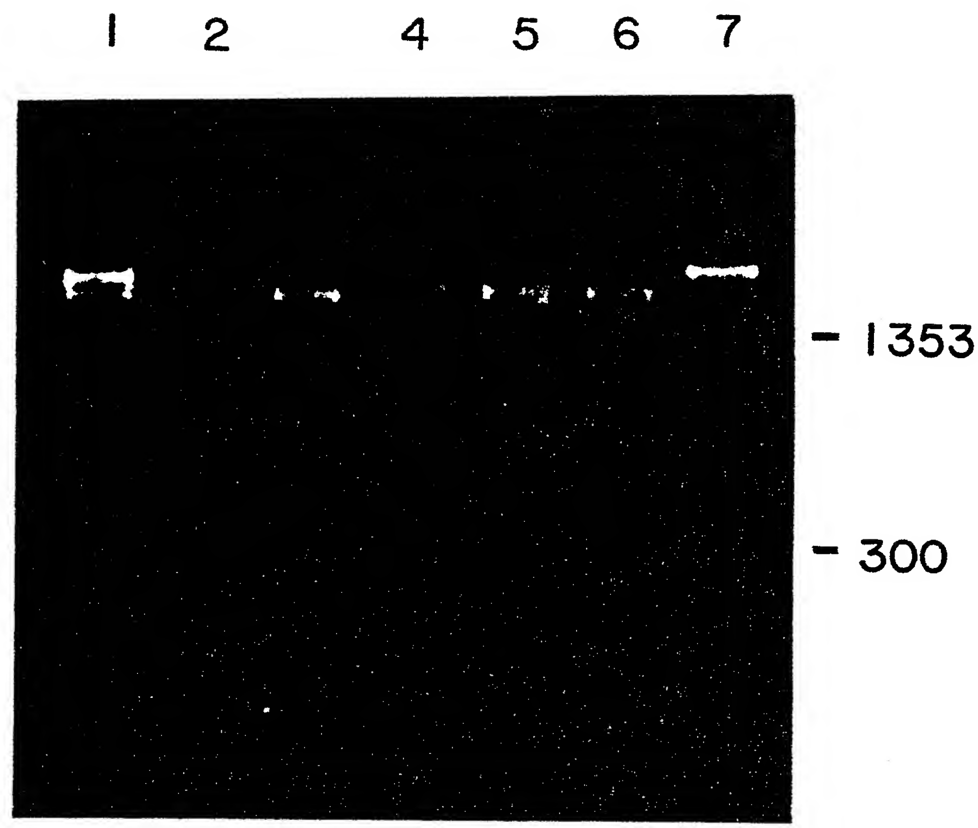


FIG. 3

pPFR-1

TGTCCTCCAGACTTTTCTACCACTCGTAGAGTTTTCTGGGTACTGTGAACTGACCTCCAGACTGATCTCT
ACAATCCGTAGAGTTTCTGGGTACTGTGAACTGTCCTCCAGACTTTTCTACCACTCGTAGAGTTTCTGGG
TACTGTGAACTGACCTCCAGACTGATCTCTACAATCCGTAGAGTTACTGGGTACTGTGAACTGACCTCC

2%

pPFR-5

TTTTAGGTTTAGGGTTCAGGGTTTAGGGTTTAGGGTTCAGGGTTTAGGTTTAGGGTTCAGGGTTCAGGGT
TCAGGGTTTAGGTTTAGGGTTCAGGGTTTAGGTTTAGGGTTCAGGGTTTAGGGTTTTCC
9%

FIG. 4

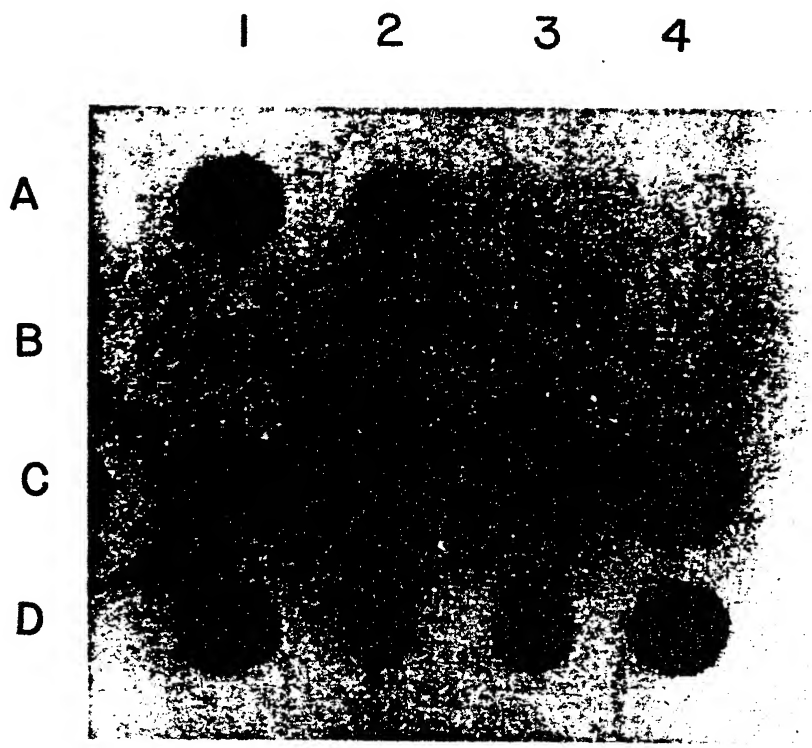


FIG. 5